

We are IntechOpen, the world's leading publisher of Open Access books Built by scientists, for scientists

4,800

Open access books available

122,000

International authors and editors

135M

Downloads

Our authors are among the

154

Countries delivered to

TOP 1%

most cited scientists

12.2%

Contributors from top 500 universities



WEB OF SCIENCE™

Selection of our books indexed in the Book Citation Index
in Web of Science™ Core Collection (BKCI)

Interested in publishing with us?
Contact book.department@intechopen.com

Numbers displayed above are based on latest data collected.
For more information visit www.intechopen.com



Applications of Anti-natural Compound Immunoaffinity Purification on Quality Control

Takuhiro Uto, Nguyen Huu Tung, Hiroyuki Tanaka
and Yukihiro Shoyama

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/45955>

1. Introduction

Worldwide demand of herbal medicines has increased in recent years owing to rising interest in the health benefits. Among with this, the quality control of plant extracts and plant-derived medicines is growing in importance to ensure their efficacy and safety. Effective quality control of the traditional Chinese medicines (TCM) and plant crude extracts requires the rapid and sensitive methods for separation and quantification of bioactive compounds. Various methods have been employed for the separation and quantification of certain constituents in medicinal plants or herbal medicines. However, the current methods in use are not necessarily optimal approaches. For example, separation and quantification of glycyrrhizin (GC), the main active constituent in licorice (*Glycyrrhiza* spp.), have been used gas chromatography, high performance liquid chromatography (HPLC) and micellar kinetic chromatography and so on [1,2]. Commercial purification of GC typically progressed through several steps, including crystallization, column chromatography, and liquid partitioning. These current methods are not sufficiently approaches because of insufficient sensitivity and reproducibility, large consumption of organic solvent for extraction and analysis, and long analysis time.

Immunoassay systems using monoclonal antibody (MAb) against drugs and small molecular weight bioactive compounds have become an important tool for studies on receptor binding assays, enzyme assays, and quantitative and qualitative analytical techniques both *in vivo* and *in vitro* studies. Although immunoaffinity purification against higher molecule analyte such as peptides and proteins are widely used in the research and commercial ways, there are too few cases of immunoaffinity purification targeting a small molecule compound such as natural compounds. Our laboratory has prepared many kinds of MAbs against

naturally occurring bioactive compounds such as terpenoids [3-5], alkaloids [6,7], saponins [8-12], and phenolics [13-16], and developed several applications. One of the applications by using MAbs is immunoaffinity column conjugated with anti-natural compound-specific MAbs and work by specifically binding and removing the target compounds. We have been establishing several affinity columns against a kind of terpenoid, forskolin [17], solasodine glycosides [18], ginsenosides Rb₁ [19], and GC [20]. Application of an immunoaffinity column to isolate and concentrate a natural compound may decrease the amount of solvent consumption and the number of purification steps, shorten analysis time, and simplify sample analysis compared to traditional cleanup techniques.

In this chapter, we focus on the immunoaffinity purification to separate and concentrate the target bioactive compounds from the crude extract. Our approaches effectively succeeded one-step purification of target compounds by MAb-conjugated immunoaffinity column, which leads to high-sensitivity detection and isolation of target compounds. In addition, the immunoaffinity column can prepare the knockout (KO) extract which contains all components except an antigen molecule, and KO extract will be useful for the pharmacological investigation to reveal the real effects of bioactive compound in the crude extract. The information in this chapter may provide new insight into quality control of plant-derived medicines.

2. Preparation of anti-ginsenoside Rb₁ immunoaffinity column and its application

Ginseng, the root of *Panax ginseng*, has been an important component in traditional medicines for more than 1000 years in Eastern Asia. It is now one of the most extensively used alternative medicines all over the world and appears in the pharmacopoeias of several countries. The biological and pharmacological activities of ginseng have been reported to have anti-aging, anti-cancer, anti-inflammation, anti-diabetics, anti-stress, maintenance of homeostasis, and to affect on central nervous system and immune function [21]. The bioactive components responsible for ginseng actions are ginsenosides, which are triterpenes saponins that possess a dammarane skeleton with sugar moieties [22]. Up to now more than 60 kinds of ginsenosides have been isolated from *Panax* genus [23]. It is well-known that the concentrations of ginsenosides vary in the ginseng root or the root extracts depending on the method of extraction, subsequent treatment, or even the season of its collection [24,25]. Due to the importance of ginseng, a number of researches has been carried out to develop the methods for the identification, quantification and quality control of ginsenosides in raw plants materials, extracts and commercial products. Currently, analytical and preparative HPLC are commonly used to quantify and purify the individual ginsenosides from ginseng [26]. However, isolation of ginsenosides by HPLC requires the repeated purification steps, including cumbersome handling and lengthy analysis times, and may result in the decrease of the final yield. Thus, the developed approaches are required for quality control of ginseng in the field of TCM.

Ginsenoside Rb₁ (G-Rb₁) is one of the main ginsenosides responsible for many pharmaceutical actions of ginseng [27]. G-Rb₁ has various biological activities, including facilitating acquisition and retrieval of memory [28], scavenging free radicals [29], inhibition of calcium over-influx into neurons [30], and preserving the structural integrity of the neurons [31].

In order to develop efficient quality control of ginseng, we have prepared anti-G-Rb₁ MAb, set up of enzyme-linked immunosorbent assay (ELISA), and a new immunostaining method named Eastern blotting [8,32]. Furthermore, we established an immunoaffinity column against G-Rb₁ and its application for one-step isolation from crude extract of ginseng root [19, 32]. Herein we describe the preparation of anti-G-Rb₁ immunoaffinity column and its applications for identification and concentration of G-Rb₁.

2.1. Preparation of MAb and immunoaffinity column against G-Rb₁

2.1.1. Analytical methodology for determination of hapten number in antigen, hapten-carrier protein conjugate

The first step for the MAb production is the synthesis of a hapten-carrier protein conjugate. Bovine serum albumin (BSA) conjugated with G-Rb₁ was produced for the preparation of specific MAb in mouse [8]. There had been no direct and appropriate methods for the determination of haptens conjugated carrier proteins without differential UV analysis, radiochemical or chemical methods. Therefore, immunization by the injection of hapten-carrier protein conjugate was unreliable. Wengatez *et al.* determined the hapten density of immuno-conjugates by matrix-assisted UV laser desorption/ionization (MALDI) mass spectrometry [33]. We also reported the direct analytical method of hapten and carrier protein conjugates by a MALDI tof mass spectrometry using internal standard [3-16]. Figure 1 shows the MALDI tof mass spectra of G-Rb₁-BSA conjugate. A broad peak coinciding with the conjugate of G-Rb₁ and BSA appeared from m/z 70,000 to 90,000 centering at around m/z 79,469. Using experimental results and a molecular weight of 66,433 for BSA, the calculated values of G-Rb₁ component (MW1,109) are from 3,327 to 23,289 resulting in the range of 3 to 21 (12 in average) molecules of G-Rb₁ conjugated with BSA [8]. This method is suitable for characterization of conjugates between small molecule natural compound and carrier protein conjugates.

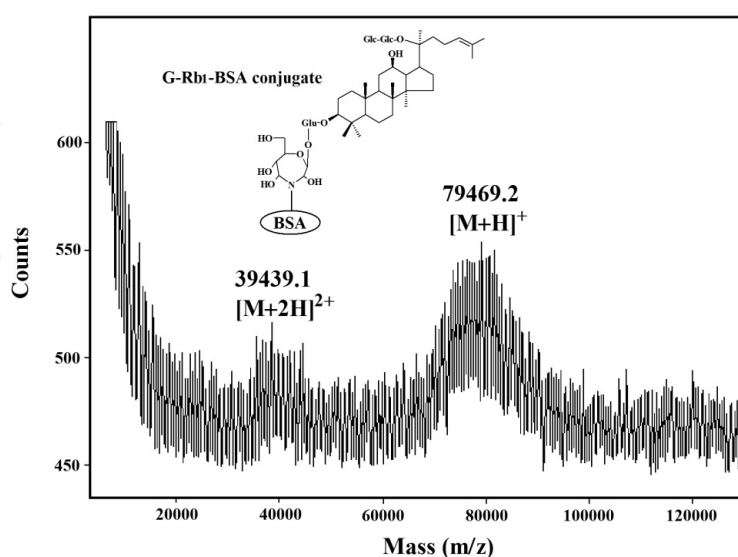


Figure 1. Direct detection of G-Rb₁-BSA conjugate by MALDI tof mass spectrometry. $[M+H]^+$, $[M+2H]^{2+}$ are single and double protonated molecules of G-Rb₁-BSA, respectively.

2.1.2. Preparation of anti-G-Rb₁ MAb and ELISA as an assay system

A hybridoma-producing MAb reactive to G-Rb₁ was obtained by general procedure and classified into IgG2b which had κ light chains [8]. The reactivity of IgG type MAb, 9G7 was tested by varying antibody concentration and by performing a dilution curve. The antibody concentration was selected for competitive ELISA. The free MAb following competition is bound to polystyrene microtiter plates precoated with G-Rb₁-human serum albumin (HSA). Under these conditions, the full measurement range of the assay extends from 20 to 400 ng/mL. The cross-reactivity against G-Rc and G-Rd, which possess a diglucose moiety attached to the C-3 hydroxy group, were weak compared with G-Rb₁ (0.024 and 0.020 %, respectively). G-Re and G-Rg₁ showed no cross-reactivity (less than 0.005 %). It is evident that the MAb reacted only with a small number of structurally related G-Rb₁ molecules, and very weakly and did not react with other steroidal compounds.

2.1.3. Preparation of anti-G-Rb₁ immunoaffinity column and appropriate buffer systems for separation of G-Rb₁

The purified IgG (10 mg) was treated by NaIO₄ to give dialdehyde group in sugar moiety which was coupled to Affi-Gel Hz hydrazide gel resulting in a hydrozone-type immunoaffinity gel [32]. The immunoaffinity gel was packed into plastic mini-column (Figure 2). Due to examine the optimal conditions of adsorption and elution, 400 μ g of G-Rb₁ was dissolved in phosphate buffered saline (PBS) and loaded on anti-G-Rb₁ affinity column. After washing with washing buffer (20 mM PB containing 0.5 M NaCl), various buffer solutions for elution were loaded on the column, and then the recovery efficiency was determined by ELISA. The G-Rb₁ concentration was somewhat increased by eluting with a 20 mM phosphate buffer containing 0.5 M KSCN and 10 % MeOH. When the 20 mM phosphate buffer was changed to 100 mM AcOH buffer (pH 4), the elution ability reached the optimal level. Although 20 % MeOH could enhance the elution of G-Rb₁, higher MeOH concentration of over 20 % was ineffective. Thus, 100 mM AcOH buffer containing 0.5 M KSCN and 20 % MeOH could be used as an elution buffer in the immunoaffinity chromatography.

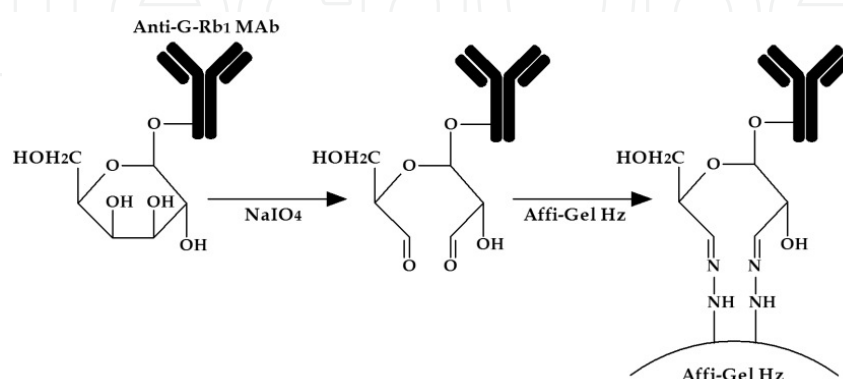


Figure 2. Preparation of anti-G-Rb₁ immunoaffinity column

2.2. Purification of G-Rb₁ by immunoaffinity column

2.2.1. One-step purification of G-Rb₁ from crude extract of *P. ginseng* roots by anti-G-Rb₁ immunoaffinity column

A crude extract (3.8 mg) of *P. ginseng* roots was loaded onto anti-G-Rb₁ immunoaffinity column. The column was washed with the washing buffer (fractions 1-20), and then eluted with elution buffer (fractions 21-40). As shown in Figure 3, the fractions 1-8 contained the overloaded G-Rb₁, which determined by ELISA. The other ginsenosides such as G-Rg₁, Rc, Re and Rd were also detected in these fractions by Eastern blotting procedure. After washing, a sharp peak was observed around fractions 21-24 of elution buffer, which contained G-Rb₁. However, these eluted fractions were still contaminated by a small amount of malonyl-G-Rb₁ as detected by Eastern blotting. The malonyl-G-Rb₁ has almost the same cross-reactivity with G-Rb₁ [32]. Therefore, the eluted fractions were treated with a mild alkaline solution (0.1 % KOH in MeOH) at room temperature to give pure G-Rb₁ [19]. Overcharged G-Rb₁ in washing solution (fractions 1-8) was repeatedly loaded and finally isolated in pure form. The anti-G-Rb₁ MAb was stable during all procedures, and the immunoaffinity column showed almost no decrease in capacity (20 µg of G-Rb₁/ml gel) after repeated use more than 10 times under same conditions, as reported for a one-step purification of forskolin from a crude extract of *Coleus forskohlii* root [17].

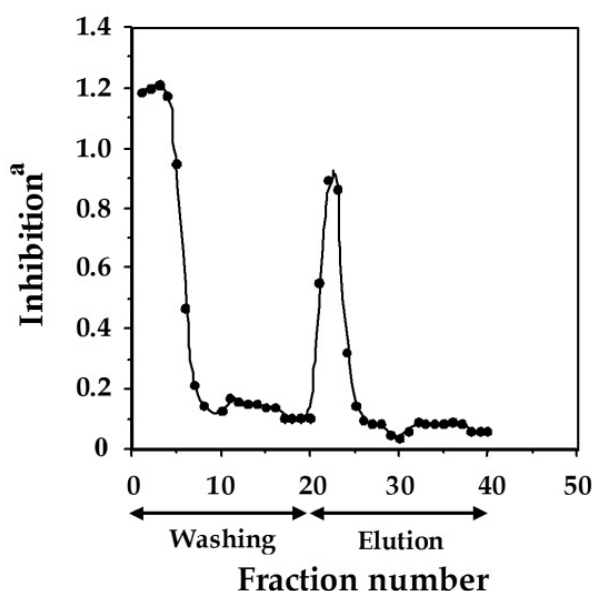


Figure 3. Elution profile of *P. ginseng* crude extract separated by anti-G-Rb₁ immunoaffinity column. The concentration of G-Rb₁ in each fraction was monitoring by ELISA using anti-G-Rb₁ MAb. Individual fraction (2 mL) were assayed by ELISA. ^aInhibition = (A₀-A)/A₀; A₀ is the absorbance in the absence of the test compounds. A is the absence in the presence the test compounds.

This methodology is effective for the rapid and simple purification of G-Rb₁ and may open up a wide field of comparable studies with other families of saponins for which an acceptable method for one-step separation has not yet been developed. Furthermore, to separate the

total ginseng saponins, a wide cross-reactive MAb against ginsenoside, like anti G-Re MAb which showed wide cross-reactivity, could be designed [34]. A combination of immunoaffinity column, Eastern blotting and ELISA could be used to survey low concentrations of ginsenoside Rb₁ of plant origin and/or in experimental animals and human. In fact we have succeeded in the isolation of G-Rb₁ from a different plant, *Kalopanax pictus* Nakai, which was not known previously to contain ginsenosides, using this combination of methods [35].

2.2.2. Isolation and determination of unknown compounds related to G-Rb₁ by anti-G-Rb₁ immunoaffinity column

Several species of ginseng are known to exist and contain different amount and kinds of ginsenosides. *P. japonicus* is distributed in Japan and China and it is morphologically different from the other *Panax* species. Yahara *et al.* indicated that G-Rb₁ was not detected in *P. japonicus*, and isolated oleanane-type saponins called chikusetsusaponins and determined their structures [36]. Morita *et al.* reported the varieties of saponins in *P. japonicus* by chemical analysis. These results suggested that the concentration of G-Rb₁ might be trace level in *P. japonicas* [37]. We previously analyzed the G-Rb₁ concentration in several ginseng roots by ELISA using anti-G-Rb₁ MAb and HPLC after pre-treatment under mildly alkaline condition [32]. As shown in Table 1, G-Rb₁ concentrations of *P. ginseng*, *P. notoginseng* and *P. quinquefolius* were correlated between ELISA and HPLC. However, the G-Rb₁ of *P. japonicus* was higher concentrations compared with HPLC and previous reports [37]. This data suggest that anti-G-Rb₁ MAb using ELISA has the cross-reactivity with some unknown compounds contained in *P. japonicus*.

Sample		G-Rb ₁ concentration (µg/mg dry weight powder)	
		ELISA	HPLC
<i>P. ginseng</i>	White ginseng	5.49 ± 0.75	4.96 ± 0.05
	Red ginseng	3.57 ± 0.62	3.93 ± 0.34
	Fibrous ginseng	64.44 ± 3.64	69.75 ± 1.45
<i>P. notoginseng</i>		47.08 ± 3.34	42.39 ± 1.39
<i>P. quinquefolium</i>		48.51 ± 1.79	47.96 ± 1.04
<i>P. japonicus</i>		1.37 ± 0.34	0.63 ± 0.06

Table 1. G-Rb₁ concentration in various ginseng samples

To clarify the unknown compounds bound to anti-G-Rb₁ MAb, the crude extract of *P. japonicus* was concentrated by immunoaffinity column using anti-G-Rb₁ MAb. The crude root extract was loaded on the column and washed with the washing Buffer, followed by the elution buffer as already indicated. Figure 4 shows the H₂SO₄ staining (A) and the Eastern blotting (B) profiles of the washing fractions 1-4 and eluted fraction 5. Fraction 1 is first elut-

ed fraction by the washing buffer, and showed many spots, indicating chikusetsusaponins, similar to the original extract of *P. japonicus*. After washing, the column was eluted by elution buffer (fraction 5), and then one spot was detected. As shown in Figure 4B, Eastern blotting indicated two different spots in washing fraction (Compound 1) and eluted fraction (Compound 2). These compounds bound with anti-G-Rb₁ MAb have a dammarane saponin having protopanaxadiol as a framework.

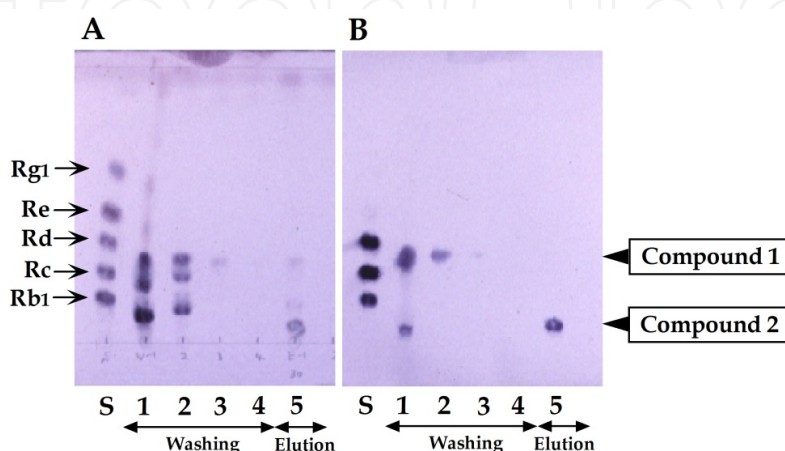


Figure 4. Purification and determination of ginsenosides of *P. japonicus* by the anti-G-Rb₁ immunoaffinity column. TLC (A) and Eastern blotting (B) profiles of the separated fractions from *P. japonicus* crude extract by the anti-G-Rb₁ immunoaffinity column. Lane S indicates the standard of ginsenosides (G-Rd, G-Rc, G-Rb₁, G-Rg₁, and G-Re). Lane 1-4 and Lane 5 were the washing fractions and the eluted fraction, respectively.

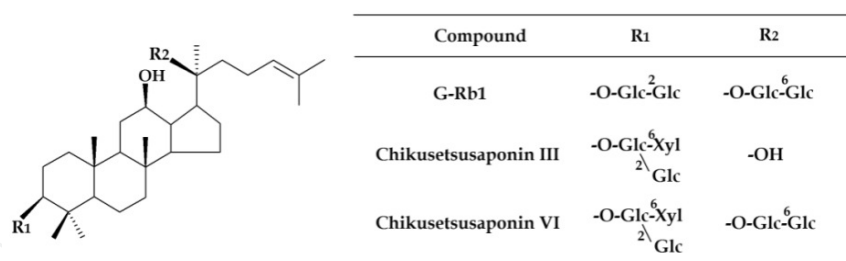


Figure 5. Chemical structures of G-Rb₁ and chikusetsusaponins purified from *P. japonicus* by the anti-G-Rb₁ immunoaffinity column.

Compound 1 has three sugar moieties in a molecule because that the R_F value closes to that of G-Rd, indicating that this compound is chikusetsusaponin III (Figure 5). Finally, we identified that this compound as chikusetsusaponin III in a direct comparison with authentic sample [32]. Another unknown spot, compound 2 appeared in fraction 5 of the eluted fraction. Thus, compound 2 has a similar molecular structure and high cross-reactivity with G-Rb₁, and seems to be related ginseng saponin having protopanaxadiol as an aglycone. Moreover, compound 2 might have the same sugar fragments and possess five sugar moiety compared with G-Rb₁, as indicated by their R_F value. From these evidences compound 2

might be chikusetsusaponin III-20-*O*-gentiobiose, chikusetsusaponin VI (Figure 5), which has 5 sugars in a molecule in good agreement with the R_F value previously reported [38] and we confirmed that compound 2 is chikusetsusaponin VI by the direct comparison with authentic sample.

These data suggested that the anti-G-Rb₁ immunoaffinity column could isolate some unknown structurally resemble compounds having cross-reactivity against anti-G-Rb₁MAB. Therefore, this purification system will be applied to survey new compounds related to target compound of MAB. In our previous studies, we demonstrated the immunoaffinity purification against all solasodine glycosides from crude extract by one-step purification. In this case, all solasodine glycoside have almost same cross-reactivity against anti-solamar-gine MAB [12].

2.2.3. Preparation of G-Rb₁ knockout extract by anti-G-Rb₁ immunoaffinity column

The capacity of this anti-G-Rb₁ immunoaffinity column is 20 µg of G-Rb₁/ml gel [32]. By loading the samples not to exceed the binding capacity against G-Rb₁, this immunoaffinity column becomes possible to remove all G-Rb₁ from crude ginseng extract. Figure 6 showed H₂SO₄ staining of TLC of the purification steps by the immunoaffinity column. Lane 1 and 2 were spotted the standard of ginsenosides (G-Rd, G-Rc, G-Rb₁, G-Rg₁, and G-Re). Lane A, B, and C were the crude extract, the washing fraction, and the eluted fraction, respectively. In the crude extract (lane A), all spots of ginsenosides were clearly detected. On the other hand, the washing fraction (lane B) contained all of the ginsenosides in the crude extract except G-Rb₁. Furthermore, the spot of G-Rb₁ was detected in the eluted fractions (lane C). These data strongly indicated that G-Rb₁ molecule in the ginseng extract can be eliminated by an anti-G-Rb₁ immunoaffinity column and the washing fractions was knockout only by the antigen molecule, G-Rb₁. Thus, we named the washing fractions a knockout (KO) extract [39,40]. This KO extract may be useful for the determination of real pharmacologically active principle in the TCMs.

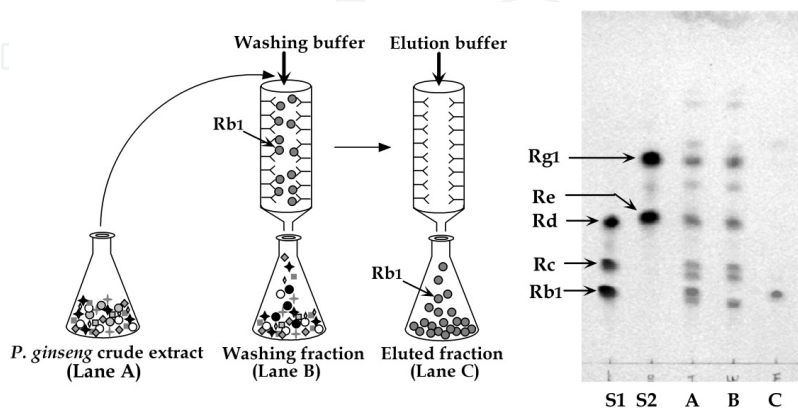


Figure 6. Preparation of G-Rb₁-KO extract from *P. ginseng* crude extract using anti-G-Rb₁ immunoaffinity column. Lane S1 and S2 indicate the standard of ginsenosides (G-Rd, G-Rc, G-Rb₁, G-Rg₁ and G-Re). Lane A, B, and C were the crude extract, the washing fraction, and the eluted fraction, respectively.

3. Glycyrrhizin-knockout extract and its application for *in vitro* assay

Licorice (*Glycyrrhiza* spp.) is also important crude drug used in over 70 % of the TCMs and Japanese Kampo medicines. It is prescribed with other herbal medicines as a demulcent in the treatment of sore throats, an expectorant for coughs and bronchial catarrh, an antitussive, a taste-modifying agent for relieving pain, an anti-inflammatory agent for anti-allergic reactions, rheumatism and arthritis, a prophylactic for liver disease and tuberculosis and adrenocorticoid insufficiency [41-43]. Accumulated evidence indicated that GC, a main saponin component of licorice, is one of the biologically active compounds. It has been reported that GC exhibits numerous pharmacological effects such as anti-inflammation, anti-ulcer, anti-tumor, anti-allergy, and hepatoprotective activities [44,45]. Clinically, GC has been used to treat patients with chronic hepatitis [46,47]. Although GC is supposed to be a major active principle in licorice crude extract, a number of studies by HPLC profiles suggested that licorice has many other bioactive components, including flavonoids, isoflavonoids and chalcones [43,48]. Biological studies showed that various flavonoid glycosides and their aglycones of licorice exhibit anti-inflammatory, anti-oxidative, anti-microbial, superoxide scavenging, and anti-carcinogenic activities [43,48]. In order to confirm the role of GC in TCM, we previously purified GC from TCM using an immunoaffinity column conjugated with anti-GC MAb [20]. In this section, we describe the preparation of GC-KO extract and its application for functional analysis of GC in licorice crude extract.

3.1. Preparation of GC-KO extract by anti-GC immunoaffinity column and the characterization of GC-KO extract

Our previous study demonstrated the preparation of anti-GC MAb [11]. The cross-reactivities of the anti-GC MAb against glycyrrhetic acid-3-O-glucuronide and glycyrrhetic acid were 0.585 % and 1.865 %, respectively. The other related compounds (deoxycholic acid, ursolic acid, and oleanolic acid) were all less than 0.005 %. Moreover, we established competitive ELISA and Eastern blotting method using anti-GC MAb [11,49].

The immunoaffinity column against GC was prepared by coupling the purified 60 mg of the anti-GC MAb to 25 ml of an Affi-Gel Hz gel [11]. To eliminate GC from licorice extract, 12 mg of licorice crude extract (GC content: 1275.0 µg) in loading buffer (5 % MeOH) was applied on the anti-GC MAb immunoaffinity column, and then the loading buffer was continuously circulated through the column to enhance the binding efficiency. After overnight circulation at 4 °C, the unbound fraction was separated. The column was washed with washing buffer (5 % MeOH) and then eluted with elution buffer (20 mM phosphate buffer containing 30 % MeOH). After separation, each fraction was deionized and the solvent was lyophilized. Figure 7 showed the recovery ratio of GC checked by ELISA. In the unbound fraction, 3.50 µg of GC (0.27% of the applied GC) was detected. On the other hand, 1269.26 µg of GC (99.55% of the applied GC) was obtained in the bound fraction. These data indicate that the anti-GC column could eliminate 99.55 % of the loading GC. Thus, we named this unbound fraction “GC-knockout (GC-KO) extract” [50].

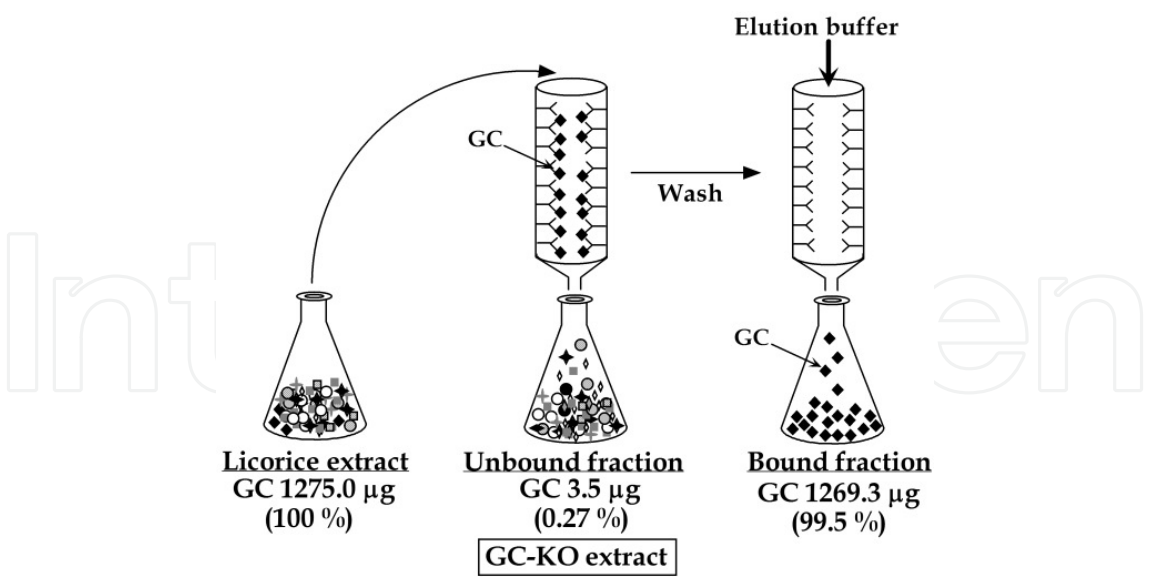


Figure 7. Preparation of GC-KO extract from licorice extract by anti-GC immunoaffinity column.

To further characterize GC-KO extract, the TLC analysis and Eastern blotting were performed [50]. As shown in Figure 8A, several spots including GC were detected in licorice extract (Lane B). However, the spot of GC was completely disappeared in GC-KO extract, although all other spots were clearly detected (lane C). Eastern blotting by anti-GC MAb indicated that GC was detected in licorice extract (Figure 8B, lane B), but the spot of GC was disappeared in GC-KO extract (Figure 8B, lane C). Therefore, these data suggest that GC was specifically eliminated from licorice extract by anti-GC MAb immunoaffinity column.

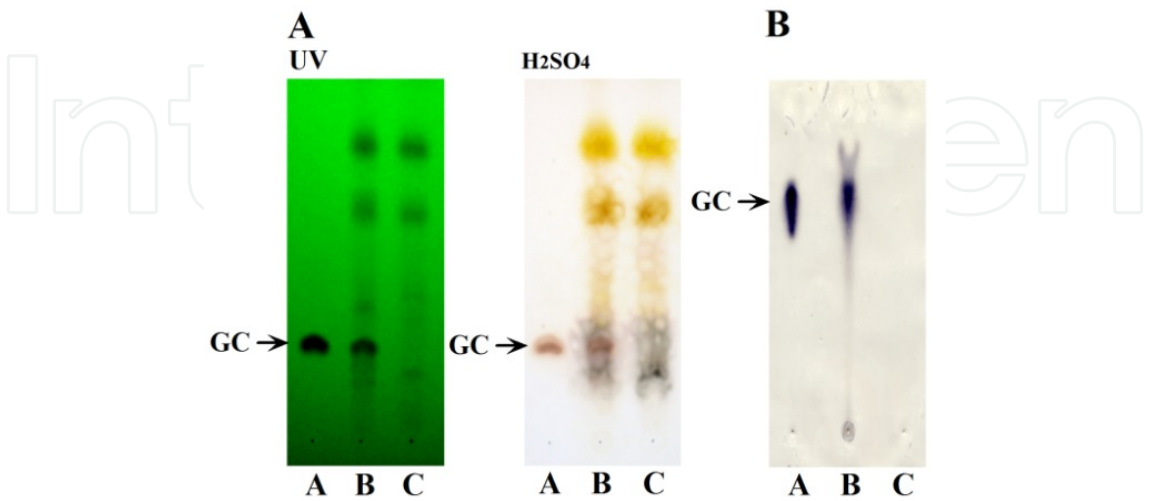


Figure 8. TLC profiles (A) and Eastern blotting by anti-GC MAb (B). Lane A, B, and C indicate GC, licorice extract, and GC-KO extract, respectively.

3.2. *in vitro* Assay by GC-KO extract prepared by anti-GC immunoaffinity column

Nitric oxide (NO), synthesized by NO synthase (NOS) from L-arginine, is an important regulatory/modulatory mediator for several physiological processes [51]. However, during inflammatory process, a large amount of NO is produced by inducible NOS (iNOS) stimulated by bacterial lipopolysaccharide (LPS) and inflammatory cytokines participates in the pathogenesis of inflammatory diseases [52]. Overproduced NO synthesized by iNOS triggers a variety of inflammatory diseases including sepsis, psoriasis, arthritis, multiple sclerosis, and systemic lupus *erythematosus* [53]. Therefore, inhibiting NO production by blocking iNOS expression may be useful strategy to treat a variety of inflammatory diseases.

In LPS-treated mouse RAW264 macrophages, licorice extract inhibited NO production and iNOS expression. At 100 µg/mL of licorice extract, iNOS protein and mRNA were completely suppressed [50].

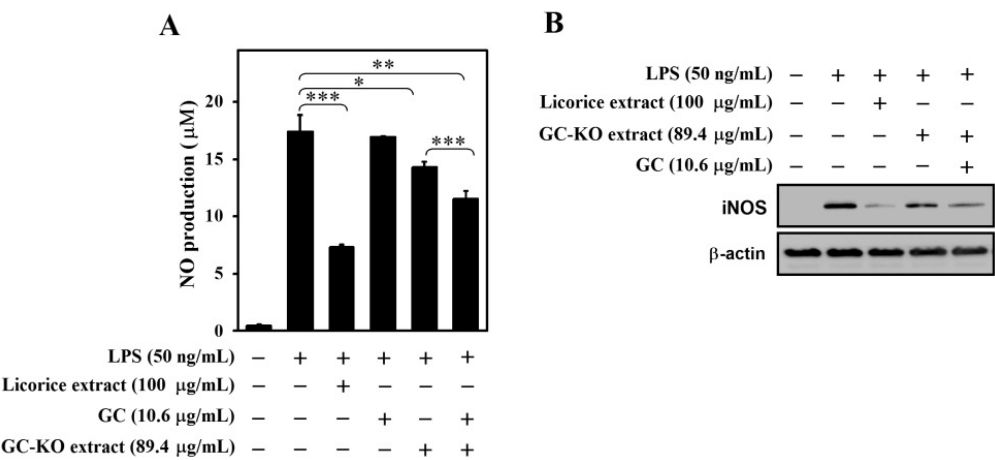


Figure 9. Effect of GC-KO extract and the combination of GC-KO extract and GC on NO production (A) and iNOS protein expression (B) in LPS-treated RAW264 cells. Each bar indicates the mean S.D. of four individual experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicate significant differences from the LPS alone

We next examined the inhibitory effect of GC alone, GC-KO extract and the combined treatment with GC and GC-KO extract on NO production [50]. Since 100 µg of licorice extract contains 10.6 ± 0.618 µg of GC, the cells were pre-treated with licorice extract (100 µg/ml), GC-KO extract (89.4 µg/ml), or the combination of GC-KO extract (89.4 µg/ml) and GC (10.6 µg/ml). Figure 9A indicated that the treatment of licorice extract led to a marked suppression of NO production as compared to LPS treatment [inhibition ratio (IR) 57.7%]. Interestingly, the inhibitory effect of GC-KO extract was lower (IR 17.8%) compared with licorice extract although GC alone could not block NO production as indicated above. On the other hand, the combined treatment with GC-KO extract and GC significantly improved the inhibitory ability (IR 33.5%). To determine whether the combinational effect of GC-KO extract and GC was related to iNOS expression, we performed Western blotting. As shown in Figure 9B, the treatment of GC-KO extract diminished the inhibitory ability of LE on iNOS expression, and addition of GC to GC-KO extract could improve it. These data suggest that GC alone cannot

suppress iNOS expression, but combinational inhibition of iNOS expression may occur when GC coexists with the other constituents contained in licorice extract. The *in vitro* and *in vivo* analysis by using KO extract prepared by immunoaffinity column is a useful approach for determination of potential function of natural compound on *in vitro* and *in vivo* assays.

4. Conclusion

In this chapter, we introduce the unique strategy of one-step purification of target compounds from crude extract by anti-natural compound specific MAb-conjugated immunoaffinity column. The immunoaffinity column conjugated with anti-G-Rb₁ MAb could purify the G-Rb₁ from *P. ginseng* extract, and the washing fraction contained all compounds except only G-Rb₁, which was named G-Rb₁-KO extract. By the use of the cross-reactivity of MAb, the anti-G-Rb₁ immunoaffinity column can identify new unknown compounds related to target compound of MAb and determine their structures. Furthermore, our data suggest that the combination of the immunoaffinity column and ELISA by using MAb provided a reliable and high sensitivity analysis for target compound in various TCMs and crude extract. We also demonstrated the *in vitro* assay by using GC-KO extract prepared by anti-GC immunoaffinity column from licorice extract. The KO extract may be able to support the pharmacological investigation for finding out a really active compound in a TCM and crude drug.

Acknowledgements

This work was funded by the Asahi Beer Science Promoting Foundation and Takeda Science Foundation. The research in this paper was also supported in part by Sasakawa Scientific Research Grant from Japan Science Society and “Science and Technology Research Partnership for Sustainable Development (SATREPS)” supported by the Japan Science and Technology Agency (JST) and the Japan International Cooperation Agency (JICA).

Author details

Takuhiro Uto¹, Nguyen Huu Tung¹, Hiroyuki Tanaka² and Yukihiro Shoyama^{1*}

*Address all correspondence to: shoyama@niu.ac.jp

1 Faculty of Pharmaceutical Sciences, Nagasaki International University,, Japan

2 Faculty of Pharmaceutical Sciences, Kyushu University,, Japan

References

- [1] Ong, E. S. (2002). Chemical assay of glycyrrhizin in medicinal plants by pressurized liquid extraction (PLE) with capillary zone electrophoresis (CZE). *J Sep Sci*, 25(13), 825-831.
- [2] Tan, T. W., Huo, Q., & Ling, Q. (2002). Purification of glycyrrhizin from glycyrrhiza uralensis fisch with ethanol/phosphate aqueous two phase system. *Biochem Lett*, 24(17), 1417-1420.
- [3] Sakata, R., Shoyama, Y., & Murakami, H. (1994). Production of monoclonal antibodies and enzyme immunoassay for typical adenylate cyclase activator, Forskolin. *Cytotechnology*, 16(2), 101-108.
- [4] Xuan, L., Tanaka, H., Xu, Y., & Shoyama, Y. (1999). Preparation of monoclonal antibody against crocin and its characterization. *Cytotechnology*, 29(1), 65-70.
- [5] Lu, Z., Morinaga, O., Tanaka, H., & Shoyama, Y. (2003). A quantitative ELISA using monoclonal antibody to survey paeoniflorin and albiflorin in crude drugs and traditional Chinese herbal medicines. *Biol Pharm Bull*, 26(6), 862-866.
- [6] Shoyama, Y., Fukada, T., & Murakami, H. (1996). Production of monoclonal antibodies and ELISA for thebaine and codeine. *Cytotechnology*, 19(1), 55-61.
- [7] Kim, J. S., Tanaka, H., & Shoyama, Y. (2004). Immunoquantitative analysis for berberine and its related compounds using monoclonal antibodies in herbal medicines. *Analyst*, 129(1), 87-91.
- [8] Tanaka, H., Fukuda, N., & Shoyama, Y. (1999). Formation of monoclonal antibody against a major ginseng component, ginsenoside Rb₁ and its characterization. *Cytotechnology*, 29(1), 115-120.
- [9] Fukuda, N., Tanaka, H., & Shoyama, Y. (2000). Formation of monoclonal antibody against a major ginseng component, ginsenoside Rg₁ and its characterization Monoclonal antibody for a ginseng saponin. *Cytotechnology*, 34(3), 197-204.
- [10] Zhu, S., Shimokawa, S., Tanaka, H., & Shoyama, Y. (2004). Development of an assay system for saikosaponin a using anti-saikosaponin a monoclonal antibodies. *Biol Pharm Bull*, 27(1), 66-71.
- [11] Shan, S. J., Tanaka, H., & Shoyama, Y. (2001). Enzyme-linked immunosorbent assay for glycyrrhizin using anti-glycyrrhizin monoclonal antibody and an eastern blotting technique for glucuronides of glycyrrhetic acid. *Anal Chem*, 73(24), 5784-5790.
- [12] Ishiyama, M., Shoyama, Y., Murakami, H., & Shinohara, H. (1996). Production of monoclonal antibodies and development of an ELISA for solamargine. *Cytotechnology*, 18(3), 153-158.

- [13] Morinaga, O., Tanaka, H., & Shoyama, Y. (2000). Production of monoclonal antibody against a major purgative component, sennoside A, its characterization and ELISA. *Analyst*, 125(8), 1109-1113.
- [14] Morinaga, O., Nakajima, S., Tanaka, H., & Shoyama, Y. (2001). Production of monoclonal antibodies against a major purgative component, sennoside B, their characterization and use in ELISA. *Analyst*, 126(8), 1372-1376.
- [15] Tanaka, H., Goto, Y., & Shoyama, Y. (1996). Monoclonal antibody based enzyme immunoassay for marihuana (cannabinoid) compounds. *Immunoassay*, 17(4), 321-342.
- [16] Loungkratana, P., Tanaka, H., & Shoyama, Y. (2004). Production of monoclonal antibody against ginkgolic acids in Ginkgo biloba Linn. *Am J Chin Med*, 32(2), 33-48.
- [17] Yanagihara, H., Sakata, R., Minami, H., Shoyama, Y., & Murakami, H. (1996). Immunoaffinity column chromatography against forskolin using an anti-forskolin monoclonal antibody and its application. *Anal Chim Acta*, 335(1-2), 63 -70.
- [18] Putalun, W., Tanaka, H., & Yukihira, S. (1999). Rapid separation of solasodine glycosides by an immunoaffinity column using anti-solamargine monoclonal antibody. *Cytotechnology*, 31(1-2), 151-156.
- [19] Fukuda, N., Tanaka, H., & Shoyama, H. (2000). Isolation of the pharmacologically active saponin ginsenoside Rb₁ from ginseng by immunoaffinity column chromatography. *J Nat Prod*, 63(2), 283-285.
- [20] Xu, J., Tanaka, H., & Shoyama, Y. (2007). One-step immunochromatographic separation and ELISA quantification of glycyrrhizin from traditional Chinese medicines. *J Chromatog B*, 850(1-2), 53-58.
- [21] Gillis, C. N. (1997). Panax ginseng pharmacology: a nitric oxide link? *Biochem Pharmacol*, 54(1), 1-8.
- [22] Liu, C. X., & Xiao, P. G. (1992). Recent advances on ginseng research in China. *J. ethnopharmacol*, 36(1), 27-38.
- [23] Yu, H., Zhang, C., Lu, M., Sun, F., Fu, Y., & Jin, F. (2007). Purification and characterization of new special ginsenosidase hydrolyzing multi-glycisides of protopanaxadiolginsenosides, ginsenosidase type I. *Chem Pharm Bull (Tokyo)*, 55(2), 231-235.
- [24] Kitagawa, I., Taniyama, T., Yoshikawa, M., Ikenishi, Y., & Nakagawa, Y. (1989). Chemical studies on crude drug processing. IV. Chemical structures of malonyl-ginsenosides Rb₁, Rb₂, Re and Rd isolated from the root of Panax Ginseng C.A. Meyer. *Chem Pharm Bull*, 37(11), 2961-2970.
- [25] Tanaka, O. (1989). Saponin-composition of Panax species. In: Shibata S, Ohtsuka Y, Saito H. (eds.) Recent advances in ginseng studies. Tokyo, Hirokawa Publishing, 43-47.
- [26] Fuzzati, N. (2004). Analysis methods of ginsenosides. *J Chromatogr B Analyt Technol Biomed Life Sci*, 812(1-2), 119 -33 .

- [27] Washida, D., & Kitanaka, S. (2003). Determination of polyacetylenes and ginsenosides in *Panax* species using high performance liquid chromatography. *Chem Pharm Bull*, 51(11), 1314-1317.
- [28] Mook-Jung, I., Hong, H. S., Boo, J. H., Lee, K. H., Yun, S. H., Cheong, M. Y., Joo, I., Huh, K., & Jung, M. W. (2001). Ginsenoside Rb₁ and Rg₁ improve spatial learning and increase hippocampal synaptophysin level in mice. *J Neurosci Res*, 63(6), 509-515.
- [29] Lim, J. H., Wen, T. C., Matsuda, S., Tanaka, J., Maeda, N., Peng, H., Aburaya, J., Ishihara, K., & Sakanaka, M. (1997). Protection of ischemic hippocampal neurons by ginsenoside Rb₁, a main ingredient of ginseng root. *Neurosci Res*, 28(3), 191-200.
- [30] Liu, M., & Zhang, J. (1995). Effects of ginsenoside Rb₁ and Rg₁ on synaptosomal free calcium level, ATPase and calmodulin in rat hippocampus. *Chin Med J (Engl)*, 108(7), 544-547.
- [31] Jiang, K. Y., & Qian, Z. N. (1995). Effects of *Panax notoginseng* saponins on posthypoxic cell damage of neurons in vitro. *Zhongguo Yao Li Xue Bao*, 16(5), 399-402.
- [32] Fukuda, N., Tanaka, H., & Shoyama, Y. (2000). Applications of ELISA, western blotting and immunoaffinity concentration for survey of ginsenosides in crude drugs of *Panax* species and traditional Chinese herbal medicines. *Analyst*, 125(8), 1425-1429.
- [33] Wengatz, I., Schmid, R. D., Kreißig, S., Wittmann, C., Hock, B., Ingendoh, A., & Hillenkamp, F. (1992). Determination of the hapten density of immuno-conjugates by matrix-assisted UV laser desorption/ionization mass spectrometry. *Anal Lett*, 25(11), 1983-1997.
- [34] Morinaga, O., Tanaka, H., & Shoyama, Y. (2006). Detection and quantification of ginsenoside Re in ginseng samples by a chromatographic immunostaining method using monoclonal antibody against ginsenoside Re. *J Chromatography B*, 830(1), 100-104.
- [35] Tanaka, H., Fukuda, N., Yahara, S., Isoda, S., Yuan, C. S., & Shoyama, Y. (2005). Isolation of ginsenoside Rb₁ from *Kalopanax pictus* by eastern blotting using anti-ginsenoside Rb₁ monoclonal antibody. *Phytother Res*, 19(3), 255-258.
- [36] Yahara, S., Kasai, R., & Tanaka, O. (1977). New dammarane type saponins of leaves of *Panax japonicus* C.A. Meyer. (1). Chikusetsusaponins L₅, L_{9a} and L₁₀. *Chem Pharm Bull*, 25(8), 2041-2047.
- [37] Morita, T., Tanaka, O., & Kohda, H. (1985). Saponin composition of rhizomes of *Panax japonicus* collected in South Kyushu, Japan, and its significance in oriental traditional medicine. *Chem Pharm Bull*, 33(9), 3852-3858.
- [38] Kohda, H., Tanaka, S., Yamaoka, Y., & Ohhara, Y. (1991). Saponins from *Amaranthus hypochondriacus*. *Chem Pharm Bull*, 39(10), 2609-2612.
- [39] Tanaka, H., Fukuda, N., & Shoyama, Y. (2007). Eastern blotting and immunoaffinity concentration using monoclonal antibody for ginseng saponins in the field of traditional chinese medicines. *J Agric Food Chem*, 55(10), 3783-3787.

- [40] Wang, C. A., & Shoyama, Y. (2006). Herbal medicine: identification, analysis, and evaluation strategies. In: Yuan CS, Bieber EJ, Bauer BA (eds) Textbook of complementary and alternative medicine, second edition. United Kingdom Informa Healthcare, 51-70.
- [41] Kim, S. C., Byun, S. H., Yang, C. H., Kim, C. Y., Kim, J. W., & Kim, S. G. (2004). Cytoprotective effects of Glycyrrhizae radix extract and its active component liquiritigenin against cadmium-induced toxicity (effects on bad translocation and cytochrome c-mediated PARP cleavage). *Toxicology*, 197(3), 239-251.
- [42] Fuchikami, J., Isohama, Y., Sakaguchi, M., Matsuda, M., Kucota, T., Akie, Y.k., Fujino, A., & Miyata, T. (2004). Effect of glycyrrhizin on late asthmatic responses induced by antigen inhalation in guinea pigs. *J Pharmacol Sci*, 94(Suppl.1), 251.
- [43] Asl, M. N., & Hosseinzadeh, H. (2008). Review of pharmacological effects of Glycyrrhiza sp. and its bioactive compounds. *Phytother Res*, 22(6), 709-724.
- [44] Jakkula, M., Boucher, T. A., Beyendorff, U., Conn, S. M., Johnson, J. E., Nolan, C. J., Peine, C. J., & Albrecht, J. H. (2004). A randomized trial of Chinese herbal medicines for the treatment of symptomatic hepatitis C. *Arch Intern Med*, 164(12), 1341-1346.
- [45] Yanagawa, Y., Ogura, M., & Fujimoto, E. (2004). Effects and cost of glycyrrhizin in the treatment of upper respiratory tract infections in members of the Japanese maritime self-defense force: Preliminary report of a prospective, randomized, double-blind, controlled, parallel-group, alternate-day treatment assignment clinical trial. *Curr Ther Res Clin Exot*, 65(1), 26-33.
- [46] Schalm, S. W., Brouwer, J. T., Bekkering, F. C., & van Rossum, T. G. (1999). New treatment strategies in non-responder patients with chronic hepatitis C. *J Hepatol*, 31(Suppl.1), 1184-1188.
- [47] Coon, J. T., & Ernst, E. (2004). Complementary and alternative therapies in the treatment of chronic hepatitis C: a systematic review. *J Hepatol*, 40(3), 491-500.
- [48] Chin, Y. W., Jung, H. A., Liu, Y., Su, B. N., Castoro, J. A., Keller, W. J., Pereira, M. A., & Kinghorn, A. D. (2007). Anti-oxidant constituents of the roots and stolons of licorice (*Glycyrrhiza glabra*). *J Agric Food Chem*, 55(12), 4691-4697.
- [49] Morinaga, O., Fujino, A., Tanaka, H., & Shoyama, Y. (2005). An on-membrane quantitative analysis system for glycyrrhizin in licorice roots and traditional Chinese medicines. *Anal Bioanal Chem*, 383(4), 668-672.
- [50] Uto, T., Morinaga, O., Tanaka, H., & Shoyama, Y. (2012). Analysis of the synergistic effect of glycyrrhizin and other constituents in licorice extract on lipopolysaccharide-induced nitric oxide production using knock-out extract. *Biochem Biophys Res Commun*, 417(1), 473-478.
- [51] Moncada, S., Palmer, R. M., & Higgs, E. A. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev*, 43(2), 109-142.

- [52] Blantz, R. C., & Munger, K. (2002). Role of nitric oxide in inflammatory conditions. *Nephron*, 90(4), 373-378.
- [53] Kröncke, K. D., Fehsel, K., & Kolb-Bachofen, V. (1998). Inducible nitric oxide synthase in human diseases. *Clin Exp Immunol*, 113(2), 147-156.

IntechOpen

IntechOpen

